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(54) Title: GENERATION OF PLANTS WITH ALTERED OIL CONTENT

(57) Abstract: The present invention is directed to plants that display an altered oil content phenotype due to altered expression of a HIO30 nucleic acid. The invention is further directed to methods of generating plants with an altered oil content phenotype.

GENERATION OF PLANTS WITH ALTERED OIL CONTENT

REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. provisional patent application no.

5   60/434,763 filed 12/18/2002, the contents of which are hereby incorporated by reference

BACKGROUND OF THE INVENTION

The ability to manipulate the composition of crop seeds, particularly the content
and composition of seed oils, has important applications in the agricultural industries,

10   relating both to processed food oils and to oils for animal feeding. Seeds of agricultural
crops contain a variety of valuable constituents, including oil, protein and starch.
Industrial processing can separate some or all of these constituents for individual sale in
specific applications. For instance, nearly 60% of the US soybean crop is crushed by the
soy processing industry. Soy processing yields purified oil, which is sold at high value,

15   while the remainder is sold principally for lower value livestock feed (US Soybean Board,
2001 Soy Stats). Canola seed is crushed to produce oil and the co-product canola meal
(Canola Council of Canada). Nearly 20% of the 1999/2000 US corn crop was industrially
refined, primarily for production of starch, ethanol and oil (Corn Refiners Association).
Thus, it is often desirable to maximize oil content of seeds. For instance, for processed

20   oilseeds such as soy and canola, increasing the absolute oil content of the seed will
increase the value of such grains. For processed corn it may be desired to either increase
or decrease oil content, depending on utilization of other major constituents. Decreasing
oil may improve the quality of isolated starch by reducing undesired flavors associated
with oil oxidation. Alternatively, in ethanol production, where flavor is unimportant,

25   increasing oil content may increase overall value. In many fed grains, such as corn and
wheat, it is desirable to increase seed oil content, because oil has higher energy content
than other seed constituents such as carbohydrate. Oilseed processing, like most grain
processing businesses, is a capital-intensive business; thus small shifts in the distribution
of products from the low valued components to the high value oil component can have

30   substantial economic impacts for grain processors.

Biotechnological manipulation of oils can provide compositional alteration and
improvement of oil yield. Compositional alterations include high oleic soybean and corn
oil (US Pat Nos 6,229,033 and 6,248,939), and laurate-containing seeds (US Pat No
5,639,790), among others. Work in compositional alteration has predominantly focused

on processed oilseeds but has been readily extendable to non-oilseed crops, including

corn. While there is considerable interest in increasing oil content, the only currently

practiced biotechnology in this area is High-Oil Corn (HOC) technology (DuPont, US Pat.

No. 5,704,160). HOC employs high oil pollinators developed by classical selection

5   breeding along with elite (male-sterile) hybrid females in a production system referred to

as TopCross. The TopCross High Oil system raises harvested grain oil content in maize

from ~3.5% to ~7%, improving the energy content of the grain.

While it has been fruitful, the HOC production system has inherent limitations.

First, the system of having a low percentage of pollinators responsible for an entire field's

10   seed set contains inherent risks, particularly in drought years. Second, oil contents in

current HOC fields have plateaued at about 9% oil. Finally, high-oil corn is not primarily

a biochemical change, but rather an anatomical mutant (increased embryo size) that has

the indirect result of increasing oil content. For these reasons, an alternative high oil

strategy, particularly one that derives from an altered biochemical output, would be

15   especially valuable.

The most obvious target crops for the processed oil market are soy and rapeseed,

and a large body of commercial work (e.g., US Pat No: 5,952,544; PCT application

WO9411516) demonstrates that *Arabidopsis* is an excellent model for oil metabolism in

these crops. Biochemical screens of seed oil composition have identified *Arabidopsis*

20   genes for many critical biosynthetic enzymes and have led to identification of

agronomically important gene orthologs. For instance, screens using chemically

mutagenized populations have identified lipid mutants whose seeds display altered fatty

acid composition (Lemieux B, et al. 1990, Theor Appl Genet 80, 234-240; James DW and

Dooner HK (1990) Theor Appl Genet 80, 241-245). T-DNA mutagenesis screens

25   (Feldmann *et al., Science* 243: 1351-1354, 1989) that detected altered fatty acid

composition identified the omega 3 desaturase (*FAD3*) and delta-12 desaturase (*FAD2*)

genes (US Pat No 5952544; Yadav NS et al. (1993) Plant Physiol 103, 467-476; Okuley et

al., Plant Cell. 1994 Jan;6(1):147-58). A screen which focused on oil content rather than

oil quality, analyzed chemically-induced mutants for wrinkled seeds or altered seed

30   density, from which altered seed oil content was inferred (Focks N and Benning C, Plant

Physiol 118:91-101, 1998). Another screen, designed to identify enzymes involved in

production of very long chain fatty acids, identified a mutation in the gene encoding a

diacylglycerol acyltransferase (DGAT) as being responsible for reduced triacyl glycerol

accumulation in seeds (Katavic V et al, Plant Physiol. 1995 May;108(1):399-409). It was

further shown that seed-specific over-expression of the DGAT cDNA was associated with increased seed oil content (Jako et al., Plant Physiol. 2001 Jun;126(2):861-74).

5   Activation tagging in plants refers to a method of generating random mutations by insertion of a heterologous nucleic acid construct comprising regulatory sequences (e.g., an enhancer) into a plant genome. The regulatory sequences can act to enhance transcription of one or more native plant genes; accordingly, activation tagging is a fruitful method for generating gain-of-function, generally dominant mutants (see, e.g., Hayashi et al., Science (1992) 258: 1350-1353; Weigel et al., Plant Physiology (2000) 122:1003-1013). The inserted construct provides a molecular tag for rapid identification of the
10   native plant whose mis-expression causes the mutant phenotype. Activation tagging may also cause loss-of-function phenotypes. The insertion may result in disruption of a native plant gene, in which case the phenotype is generally recessive.

Activation tagging has been used in various species, including tobacco and Arabidopsis, to identify many different kinds of mutant phenotypes and the genes
15   associated with these phenotypes (Wilson et al., Plant Cell (1996) 8:659-671, Schaffer et al., Cell (1998) 93: 1219-1229; Fridborg et al., Plant Cell (1999)11: 1019-1032; Kardailsky et al., Science (1999) 286:1962-1965); Christensen S et al., 9th International Conference on Arabidopsis Research. Univ. of Wisconsin-Madison, June 24-28, 1998. Abstract 165).

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SUMMARY OF THE INVENTION

The invention provides a transgenic plant having a high oil phenotype. The transgenic plant comprises a transformation vector comprising a nucleotide sequence that encodes or is complementary to a sequence that encodes a HIO30 polypeptide. In
25   preferred embodiments, the transgenic plant is selected from the group consisting of rapeseed, soy, corn, sunflower, cotton, cocoa, safflower, oil palm, coconut palm, flax, castor and peanut. The invention further provides a method of producing oil comprising growing the transgenic plant and recovering oil from said plant.

The transgenic plant of the invention is produced by a method that comprises
30   introducing into progenitor cells of the plant a plant transformation vector comprising a nucleotide sequence that encodes or is complementary to a sequence that encodes a HIO30 polypeptide, and growing the transformed progenitor cells to produce a transgenic plant, wherein the HIO30 polynucleotide sequence is expressed causing the high oil phenotype.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Unless otherwise indicated, all technical and scientific terms used herein have the same meaning as they would to one skilled in the art of the present invention.

5   Practitioners are particularly directed to Sambrook *et al.* Molecular Cloning: A Laboratory Manual (Second Edition), Cold Spring Harbor Press, Plainview, N.Y.,1989, and Ausubel FM *et al.* Current Protocols in Molecular Biology, John Wiley & Sons, New York, N.Y., 1993, for definitions and terms of the art. It is to be understood that this invention is not limited to the particular methodology, protocols, and reagents described, as these may

10   vary.

As used herein, the term "vector" refers to a nucleic acid construct designed for transfer between different host cells. An "expression vector" refers to a vector that has the ability to incorporate and express heterologous DNA fragments in a foreign cell. Many prokaryotic and eukaryotic expression vectors are commercially available. Selection of

15   appropriate expression vectors is within the knowledge of those having skill in the art.

A "heterologous" nucleic acid construct or sequence has a portion of the sequence that is not native to the plant cell in which it is expressed. Heterologous, with respect to a control sequence refers to a control sequence (*i.e.* promoter or enhancer) that does not function in nature to regulate the same gene the expression of which it is currently

20   regulating. Generally, heterologous nucleic acid sequences are not endogenous to the cell or part of the genome in which they are present, and have been added to the cell, by infection, transfection, microinjection, electroporation, or the like. A "heterologous" nucleic acid construct may contain a control sequence/DNA coding sequence combination that is the same as, or different from a control sequence/DNA coding sequence

25   combination found in the native plant.

As used herein, the term "gene" means the segment of DNA involved in producing a polypeptide chain, which may or may not include regions preceding and following the coding region, *e.g.* 5' untranslated (5' UTR) or "leader" sequences and 3' UTR or "trailer" sequences, as well as intervening sequences (introns) between individual coding segments

30   (exons) and non-transcribed regulatory sequence.

As used herein, "recombinant" includes reference to a cell or vector, that has been modified by the introduction of a heterologous nucleic acid sequence or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found in identical form within the native (non-recombinant) form of the cell or

express native genes that are otherwise abnormally expressed, under expressed or not expressed at all as a result of deliberate human intervention.

As used herein, the term "gene expression" refers to the process by which a polypeptide is produced based on the nucleic acid sequence of a gene. The process

5   includes both transcription and translation; accordingly, "expression" may refer to either a polynucleotide or polypeptide sequence, or both. Sometimes, expression of a polynucleotide sequence will not lead to protein translation. "Over-expression" refers to increased expression of a polynucleotide and/or polypeptide sequence relative to its expression in a wild-type (or other reference [e.g., non-transgenic]) plant and may relate to

10   a naturally-occurring or non-naturally occurring sequence. "Ectopic expression" refers to expression at a time, place, and/or increased level that does not naturally occur in the non-altered or wild-type plant. "Under-expression" refers to decreased expression of a polynucleotide and/or polypeptide sequence, generally of an endogenous gene, relative to its expression in a wild-type plant. The terms "mis-expression" and "altered expression"

15   encompass over-expression, under-expression, and ectopic expression.

The term "introduced" in the context of inserting a nucleic acid sequence into a cell, means "transfection", or "transformation" or "transduction" and includes reference to the incorporation of a nucleic acid sequence into a eukaryotic or prokaryotic cell where the nucleic acid sequence may be incorporated into the genome of the cell (for example,

20   chromosome, plasmid, plastid, or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (for example, transfected mRNA).

As used herein, a "plant cell" refers to any cell derived from a plant, including cells from undifferentiated tissue (*e.g.*, callus) as well as plant seeds, pollen, progagules and embryos.

25   As used herein, the terms "native" and "wild-type" relative to a given plant trait or phenotype refers to the form in which that trait or phenotype is found in the same variety of plant in nature.

As used herein, the term "modified" regarding a plant trait, refers to a change in the phenotype of a transgenic plant relative to the similar non-transgenic plant. An "altered

30   oil content phenotype" refers to measurable phenotype of a genetically modified plant, where the plant displays a statistically significant increase or decrease in overall oil content (i.e., the percentage of seed mass that is oil), as compared to the similar, but non-modified plant. A high oil phenotype refers to an increase in overall oil content.

As used herein, a "mutant" polynucleotide sequence or gene differs from the corresponding wild type polynucleotide sequence or gene either in terms of sequence or expression, where the difference contributes to a modified plant phenotype or trait. Relative to a plant or plant line, the term "mutant" refers to a plant or plant line which has
5   a modified plant phenotype or trait, where the modified phenotype or trait is associated with the modified expression of a wild type polynucleotide sequence or gene.

As used herein, the term "T1" refers to the generation of plants from the seed of T0 plants. The T1 generation is the first set of transformed plants that can be selected by application of a selection agent, *e.g.*, an antibiotic or herbicide, for which the transgenic
10   plant contains the corresponding resistance gene. The term "T2" refers to the generation of plants by self-fertilization of the flowers of T1 plants, previously selected as being transgenic. T3 plants are generated from T2 plants, etc. As used herein, the "direct progeny" of a given plant derives from the seed (or, sometimes, other tissue) of that plant and is in the immediately subsequent generation; for instance, for a given lineage, a T2
15   plant is the direct progeny of a T1 plant. The "indirect progeny" of a given plant derives from the seed (or other tissue) of the direct progeny of that plant, or from the seed (or other tissue) of subsequent generations in that lineage; for instance, a T3 plant is the indirect progeny of a T1 plant.

As used herein, the term "plant part" includes any plant organ or tissue, including,
20   without limitation, seeds, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores. Plant cells can be obtained from any plant organ or tissue and cultures prepared therefrom. The class of plants which can be used in the methods of the present invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledenous
25   and dicotyledenous plants.

As used herein, "transgenic plant" includes a plant that comprises within its genome a heterologous polynucleotide. The heterologous polynucleotide can be either stably integrated into the genome, or can be extra-chromosomal. Preferably, the polynucleotide of the present invention is stably integrated into the genome such that the
30   polynucleotide is passed on to successive generations. A plant cell, tissue, organ, or plant into which the heterologous polynucleotides have been introduced is considered "transformed", "transfected", or "transgenic". Direct and indirect progeny of transformed plants or plant cells that also contain the heterologous polynucleotide are also considered transgenic.

Identification of Plants with an Altered Oil Content Phenotype

We used an *Arabidopsis* activation tagging screen to identify the association between the gene we have designated "HIO30," (At3g52260; GI#22331747:54-938), and an altered oil content phenotype (specifically, a high oil phenotype). Briefly, and as

5   further described in the Examples, a large number of *Arabidopsis* plants were mutated with the pSKI015 vector, which comprises a T-DNA from the Ti plasmid of *Agrobacterium tumifaciens*, a viral enhancer element, and a selectable marker gene (Weigel *et al, supra*). When the T-DNA inserts into the genome of transformed plants, the enhancer element can cause up-regulation genes in the vicinity, generally within about 10

10   kilobase (kb) of the insertion. T1 plants were exposed to the selective agent in order to specifically recover transformed plants that expressed the selectable marker and therefore harbored T-DNA insertions. Samples of approximately 15-20 T2 seeds were collected from transformed T1 plants, and lipids were extracted from whole seeds. Gas chromatography (GC) analysis was performed to determine fatty acid content and

15   composition of seed samples.

An *Arabidopsis* line that showed a high-oil phenotype was identified, wherein oils (i.e., fatty acids) constituted approximately 35% of seed mass. The association of the HIO30 gene with the high oil phenotype was discovered by analysis of the genomic DNA sequence flanking the T-DNA insertion in the identified line. Accordingly, HIO30 genes

20   and/or polypeptides may be employed in the development of genetically modified plants having a modified oil content phenotype ("a HIO30 phenotype"). HIO30 genes may be used in the generation of oilseed crops that provide improved oil yield from oilseed processing and in the generation of feed grain crops that provide increased energy for animal feeding. HIO30 genes may further be used to increase the oil content of specialty

25   oil crops, in order to augment yield of desired unusual fatty acids. Transgenic plants that have been genetically modified to express HIO30 can be used in the production of oil, wherein the transgenic plants are grown, and oil is obtained from plant parts (e.g. seed) using standard methods.

30   ## HIO30 Nucleic Acids and Polypeptides

Arabidopsis HIO30 nucleic acid (genomic DNA) sequence is provided in SEQ ID NO:1 and in Genbank entry GI#22331747:54-938. The corresponding protein sequence is provided in SEQ ID NO:2 and in GI#22331748. Nucleic acids and/or proteins that are orthologs or paralogs of *Arabidopsis* HIO30, are described in Example 3 below.

As used herein, the term "HIO30 polypeptide" refers to a full-length HIO30 protein or a fragment, derivative (variant), or ortholog thereof that is "functionally active," meaning that the protein fragment, derivative, or ortholog exhibits one or more or the functional activities associated with the polypeptide of SEQ ID NO:2. In one preferred

5   embodiment, a functionally active HIO30 polypeptide causes an altered oil content phenotype when mis-expressed in a plant. In a further preferred embodiment, mis-expression of the HIO30 polypeptide causes a high oil phenotype in a plant. In another embodiment, a functionally active HIO30 polypeptide is capable of rescuing defective (including deficient) endogenous HIO30 activity when expressed in a plant or in plant

10   cells; the rescuing polypeptide may be from the same or from a different species as that with defective activity. In another embodiment, a functionally active fragment of a full length HIO30 polypeptide (i.e., a native polypeptide having the sequence of SEQ ID NO:2 or a naturally occurring ortholog thereof) retains one of more of the biological properties associated with the full-length HIO30 polypeptide, such as signaling activity, binding

15   activity, catalytic activity, or cellular or extra-cellular localizing activity. A HIO30 fragment preferably comprises a HIO30 domain, such as a C- or N-terminal or catalytic domain, among others, and preferably comprises at least 10, preferably at least 20, more preferably at least 25, and most preferably at least 50 contiguous amino acids of a HIO30 protein. Functional domains can be identified using the PFAM program (Bateman A et

20   al., 1999 Nucleic Acids Res 27:260-262; website at pfam.wustl.edu). A preferred HIO30 fragment comprises a pseudouridylate synthase domain (PFam00849). Functionally active variants of full-length HIO30 polypeptides or fragments thereof include polypeptides with amino acid insertions, deletions, or substitutions that retain one of more of the biological properties associated with the full-length HIO30 polypeptide. In some cases, variants are

25   generated that change the post-translational processing of a HIO30 polypeptide. For instance, variants may have altered protein transport or protein localization characteristics or altered protein half-life compared to the native polypeptide.

As used herein, the term "HIO30 nucleic acid" encompasses nucleic acids with the sequence provided in or complementary to the sequence provided in SEQ ID NO:1, as

30   well as functionally active fragments, derivatives, or orthologs thereof. A HIO30 nucleic acid of this invention may be DNA, derived from genomic DNA or cDNA, or RNA.

In one embodiment, a functionally active HIO30 nucleic acid encodes or is complementary to a nucleic acid that encodes a functionally active HIO30 polypeptide. Included within this definition is genomic DNA that serves as a template for a primary

RNA transcript (i.e., an mRNA precursor) that requires processing, such as splicing, before encoding the functionally active HIO30 polypeptide. A HIO30 nucleic acid can include other non-coding sequences, which may or may not be transcribed; such sequences include 5' and 3' UTRs, polyadenylation signals and regulatory sequences that control

5   gene expression, among others, as are known in the art. Some polypeptides require processing events, such as proteolytic cleavage, covalent modification, etc., in order to become fully active. Accordingly, functionally active nucleic acids may encode the mature or the pre-processed HIO30 polypeptide, or an intermediate form. A HIO30 polynucleotide can also include heterologous coding sequences, for example, sequences

10   that encode a marker included to facilitate the purification of the fused polypeptide, or a transformation marker.

In another embodiment, a functionally active HIO30 nucleic acid is capable of being used in the generation of loss-of-function HIO30 phenotypes, for instance, via antisense suppression, co-suppression, etc.

15   In one preferred embodiment, a HIO30 nucleic acid used in the methods of this invention comprises a nucleic acid sequence that encodes or is complementary to a sequence that encodes a HIO30 polypeptide having at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95% or more sequence identity to the polypeptide sequence presented in SEQ ID NO:2.

20   In another embodiment a HIO30 polypeptide of the invention comprises a polypeptide sequence with at least 50% or 60% identity to the HIO30 polypeptide sequence of SEQ ID NO:2, and may have at least 70%, 80%, 85%, 90% or 95% or more sequence identity to the HIO30 polypeptide sequence of SEQ ID NO:2. In another embodiment, a HIO30 polypeptide comprises a polypeptide sequence with at least 50%,

25   60%, 70%, 80%, 85%, 90% or 95% or more sequence identity to a functionally active fragment of the polypeptide presented in SEQ ID NO:2, such as a pseudouridylate synthase domain. In yet another embodiment, a HIO30 polypeptide comprises a polypeptide sequence with at least 50%, 60 %, 70%, 80%, or 90% identity to the polypeptide sequence of SEQ ID NO:2 over its entire length and comprises a pseudouridylate synthase domain.

30   In another aspect, a HIO30 polynucleotide sequence is at least 50% to 60% identical over its entire length to the HIO30 nucleic acid sequence presented as SEQ ID NO:1, or nucleic acid sequences that are complementary to such a HIO30 sequence, and may comprise at least 70%, 80%, 85%, 90% or 95% or more sequence identity to the

HIO30 sequence presented as SEQ ID NO:1 or a functionally active fragment thereof, or complementary sequences.

As used herein, "percent (%) sequence identity" with respect to a specified subject sequence, or a specified portion thereof, is defined as the percentage of nucleotides or amino acids in the candidate derivative sequence identical with the nucleotides or amino acids in the subject sequence (or specified portion thereof), after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent sequence identity, as generated by the program WU-BLAST-2.0a19 (Altschul et al., J. Mol. Biol. (1997) 215:403-410; website at blast.wustl.edu/blast/README.html) with search parameters set to default values. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched. A "% identity value" is determined by the number of matching identical nucleotides or amino acids divided by the sequence length for which the percent identity is being reported. "Percent (%) amino acid sequence similarity" is determined by doing the same calculation as for determining % amino acid sequence identity, but including conservative amino acid substitutions in addition to identical amino acids in the computation. A conservative amino acid substitution is one in which an amino acid is substituted for another amino acid having similar properties such that the folding or activity of the protein is not significantly affected. Aromatic amino acids that can be substituted for each other are phenylalanine, tryptophan, and tyrosine; interchangeable hydrophobic amino acids are leucine, isoleucine, methionine, and valine; interchangeable polar amino acids are glutamine and asparagine; interchangeable basic amino acids are arginine, lysine and histidine; interchangeable acidic amino acids are aspartic acid and glutamic acid; and interchangeable small amino acids are alanine, serine, threonine, cysteine and glycine.

Derivative nucleic acid molecules of the subject nucleic acid molecules include sequences that selectively hybridize to the nucleic acid sequence of SEQ ID NO:1. The stringency of hybridization can be controlled by temperature, ionic strength, pH, and the presence of denaturing agents such as formamide during hybridization and washing. Conditions routinely used are well known (see, e.g., Current Protocol in Molecular Biology, Vol. 1, Chap. 2.10, John Wiley & Sons, Publishers (1994); Sambrook et al., Molecular Cloning, Cold Spring Harbor (1989)). In some embodiments, a nucleic acid molecule of the invention is capable of hybridizing to a nucleic acid molecule containing

the nucleotide sequence of SEQ ID NO:1 under stringent hybridization conditions that are: prehybridization of filters containing nucleic acid for 8 hours to overnight at 65° C in a solution comprising 6X single strength citrate (SSC) (1X SSC is 0.15 M NaCl, 0.015 M Na citrate; pH 7.0), 5X Denhardt's solution, 0.05% sodium pyrophosphate and 100 μg/ml

5   herring sperm DNA; hybridization for 18-20 hours at 65° C in a solution containing 6X SSC, 1X Denhardt's solution, 100 μg/ml yeast tRNA and 0.05% sodium pyrophosphate; and washing of filters at 65° C for 1 h in a solution containing 0.1X SSC and 0.1% SDS (sodium dodecyl sulfate). In other embodiments, moderately stringent hybridization conditions are used that are: pretreatment of filters containing nucleic acid for 6 h at 40° C

10 in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 μg/ml denatured salmon sperm DNA; hybridization for 18-20 h at 40° C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 μg/ml salmon sperm DNA, and 10% (wt/vol) dextran sulfate; followed by washing twice for 1

15 hour at 55° C in a solution containing 2X SSC and 0.1% SDS. Alternatively, low stringency conditions can be used that comprise: incubation for 8 hours to overnight at 37° C in a solution comprising 20% formamide, 5 x SSC, 50 mM sodium phosphate (pH 7.6), 5X Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured sheared salmon sperm DNA; hybridization in the same buffer for 18 to 20 hours; and washing of filters in

20 1 x SSC at about 37° C for 1 hour.

As a result of the degeneracy of the genetic code, a number of polynucleotide sequences encoding a HIO30 polypeptide can be produced. For example, codons may be selected to increase the rate at which expression of the polypeptide occurs in a particular host species, in accordance with the optimum codon usage dictated by the particular host

25 organism (see, e.g., Nakamura et al, 1999, Nucleic Acids Res 27:292). Such sequence variants may be used in the methods of this invention.

The methods of the invention may use orthologs of the *Arabidopsis* HIO30. Methods of identifying the orthologs in other plant species are known in the art. Normally, orthologs in different species retain the same function, due to presence of one

30 or more protein motifs and/or 3-dimensional structures. In evolution, when a gene duplication event follows speciation, a single gene in one species, such as *Arabidopsis*, may correspond to multiple genes (paralogs) in another. As used herein, the term "orthologs" encompasses paralogs. When sequence data is available for a particular plant species, orthologs are generally identified by sequence homology analysis, such as

BLAST analysis, usually using protein bait sequences. Sequences are assigned as a potential ortholog if the best hit sequence from the forward BLAST result retrieves the original query sequence in the reverse BLAST (Huynen MA and Bork P, Proc Natl Acad Sci (1998) 95:5849-5856; Huynen MA *et al.*, Genome Research (2000) 10:1204-1210).

5   Programs for multiple sequence alignment, such as CLUSTAL (Thompson JD et al, 1994, Nucleic Acids Res 22:4673-4680) may be used to highlight conserved regions and/or residues of orthologous proteins and to generate phylogenetic trees. In a phylogenetic tree representing multiple homologous sequences from diverse species (e.g., retrieved through BLAST analysis), orthologous sequences from two species generally appear closest on the

10   tree with respect to all other sequences from these two species. Structural threading or other analysis of protein folding (e.g., using software by ProCeryon, Biosciences, Salzburg, Austria) may also identify potential orthologs. Nucleic acid hybridization methods may also be used to find orthologous genes and are preferred when sequence data are not available. Degenerate PCR and screening of cDNA or genomic DNA libraries are

15   common methods for finding related gene sequences and are well known in the art (see, e.g., Sambrook, *supra*; Dieffenbach C and Dveksler G (Eds.) PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY, 1989). For instance, methods for generating a cDNA library from the plant species of interest and probing the library with partially homologous gene probes are described in Sambrook *et al, supra*. A highly

20   conserved portion of the *Arabidopsis* HIO30 coding sequence may be used as a probe. HIO30 ortholog nucleic acids may hybridize to the nucleic acid of SEQ ID NO:1 under high, moderate, or low stringency conditions. After amplification or isolation of a segment of a putative ortholog, that segment may be cloned and sequenced by standard techniques and utilized as a probe to isolate a complete cDNA or genomic clone.

25   Alternatively, it is possible to initiate an EST project to generate a database of sequence information for the plant species of interest. In another approach, antibodies that specifically bind known HIO30 polypeptides are used for ortholog isolation (see, e.g., Harlow E and Lane D, Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1999, New York). Western blot analysis can determine that a HIO30

30   ortholog (i.e., an orthologous protein) is present in a crude extract of a particular plant species. When reactivity is observed, the sequence encoding the candidate ortholog may be isolated by screening expression libraries representing the particular plant species. Expression libraries can be constructed in a variety of commercially available vectors, including lambda gt11, as described in Sambrook, *et al., supra*. Once the candidate

ortholog(s) are identified by any of these means, candidate orthologous sequence are used as bait (the "query") for the reverse BLAST against sequences from *Arabidopsis* or other species in which HIO30 nucleic acid and/or polypeptide sequences have been identified.

5   HIO30 nucleic acids and polypeptides may be obtained using any available method. For instance, techniques for isolating cDNA or genomic DNA sequences of interest by screening DNA libraries or by using polymerase chain reaction (PCR), as previously described, are well known in the art. Alternatively, nucleic acid sequence may be synthesized. Any known method, such as site directed mutagenesis (Kunkel TA *et al.*, *Methods Enzymol.* 204:125-39, 1991), may be used to introduce desired changes into a
10   cloned nucleic acid.

       In general, the methods of the invention involve incorporating the desired form of the HIO30 nucleic acid into a plant expression vector for transformation of in plant cells, and the HIO30 polypeptide is expressed in the host plant.

       An isolated HIO30 nucleic acid molecule is other than in the form or setting in
15   which it is found in nature and is identified and separated from least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the HIO30 nucleic acid. However, an isolated HIO30 nucleic acid molecule includes HIO30 nucleic acid molecules contained in cells that ordinarily express HIO30 where, for example, the nucleic acid molecule is in a chromosomal location different from that of
20   natural cells.

Generation of Genetically Modified Plants with an Altered Oil Content Phenotype

       HIO30 nucleic acids and polypeptides may be used in the generation of genetically modified plants having a modified oil content phenotype. As used herein, a "modified oil content phenotype" may refer to modified oil content in any part of the plant; the modified
25   oil content is often observed in seeds. In a preferred embodiment, altered expression of the HIO30 gene in a plant is used to generate plants with a high oil phenotype.

       The methods described herein are generally applicable to all plants. Although activation tagging and gene identification is carried out in *Arabidopsis*, the HIO30 gene
30   (or an ortholog, variant or fragment thereof) may be expressed in any type of plant. In a preferred embodiment, the invention is directed to oil-producing plants, which produce and store triacylglycerol in specific organs, primarily in seeds. Such species include soybean (*Glycine max*), rapeseed and canola (including *Brassica napus, B. campestris*), sunflower (*Helianthus annus*), cotton (*Gossypium hirsutum*), corn (*Zea mays*), cocoa

(*Theobroma cacao*), safflower (*Carthamus tinctorius*), oil palm (*Elaeis guineensis*), coconut palm (*Cocos nucifera*), flax (*Linum usitatissimum*), castor (*Ricinus communis*) and peanut (*Arachis hypogaea*). The invention may also be directed to fruit- and vegetable-bearing plants, grain-producing plants, nut-producing plants, rapid cycling

5   *Brassica* species, alfalfa (Medicago sativa), tobacco (Nicotiana), turfgrass (Poaceae family), other forage crops, and wild species that may be a source of unique fatty acids.

The skilled artisan will recognize that a wide variety of transformation techniques exist in the art, and new techniques are continually becoming available. Any technique that is suitable for the target host plant can be employed within the scope of the present

10   invention. For example, the constructs can be introduced in a variety of forms including, but not limited to as a strand of DNA, in a plasmid, or in an artificial chromosome. The introduction of the constructs into the target plant cells can be accomplished by a variety of techniques, including, but not limited to *Agrobacterium*-mediated transformation, electroporation, microinjection, microprojectile bombardment calcium-phosphate-DNA

15   co-precipitation or liposome-mediated transformation of a heterologous nucleic acid. The transformation of the plant is preferably permanent, *i.e.* by integration of the introduced expression constructs into the host plant genome, so that the introduced constructs are passed onto successive plant generations. Depending upon the intended use, a heterologous nucleic acid construct comprising an HIO30 polynucleotide may encode the

20   entire protein or a biologically active portion thereof.

In one embodiment, binary Ti-based vector systems may be used to transfer polynucleotides. Standard *Agrobacterium* binary vectors are known to those of skill in the art, and many are commercially available (e.g., pBI121 Clontech Laboratories, Palo Alto, CA).

25   The optimal procedure for transformation of plants with *Agrobacterium* vectors will vary with the type of plant being transformed. Exemplary methods for *Agrobacterium*-mediated transformation include transformation of explants of hypocotyl, shoot tip, stem or leaf tissue, derived from sterile seedlings and/or plantlets. Such transformed plants may be reproduced sexually, or by cell or tissue culture.

30   *Agrobacterium* transformation has been previously described for a large number of different types of plants and methods for such transformation may be found in the scientific literature. Of particular relevance are methods to transform commercially important crops, such as rapeseed (De Block et al., Plant Physiol. (1989) 91:694-701), sunflower (Everett et

al., Bio/Technology (1987) 5:1201), and soybean (Christou et al., Proc. Natl. Acad. Sci USA (1989) 86:7500-7504; Kline et al., Nature (1987) 327:70).

Expression (including transcription and translation) of HIO30 may be regulated with respect to the level of expression, the tissue type(s) where expression takes place and/or developmental stage of expression. A number of heterologous regulatory sequences (e.g., promoters and enhancers) are available for controlling the expression of a HIO30 nucleic acid. These include constitutive, inducible and regulatable promoters, as well as promoters and enhancers that control expression in a tissue- or temporal-specific manner. Exemplary constitutive promoters include the raspberry E4 promoter (U.S. Patent Nos. 5,783,393 and 5,783,394), the 35S CaMV (Jones JD *et al*, Transgenic Res 1:285-297 1992), the CsVMV promoter (Verdaguer B *et al.*, Plant Mol Biol 37:1055-1067, 1998) and the melon actin promoter (published PCT application WO0056863). Exemplary tissue-specific promoters include the tomato E4 and E8 promoters (U.S. Pat. No. 5,859,330) and the tomato 2AII gene promoter (Van Haaren MJJ *et al.*, Plant Mol Bio 21:625-640, 1993).

In one preferred embodiment, HIO30 expression is under control of regulatory sequences from genes whose expression is associated with early seed and/or embryo development. Legume genes whose promoters are associated with early seed and embryo development include *V. faba legumin* (Baumlein et al., 1991, Mol Gen Genet 225:121-8; Baumlein et al., 1992, Plant J 2:233-9), *V. faba usp* (Fiedler et al., 1993, Plant Mol Biol 22:669-79), pea *convicilin* (Bown et al., 1988, Biochem J 251:717-26), pea *lectin* (dePater et al., 1993, Plant Cell 5:877-86), *P. vulgaris beta phaseolin* (Bustos et al., 1991, EMBO J 10:1469-79), *P. vulgaris DLEC2* and *PHS* [beta] (Bobb et al, 1997, Nucleic Acids Res 25:641-7), and soybean *beta-Conglycinin*, 7S storage protein (Chamberland et al., 1992, Plant Mol Biol 19:937-49). Cereal genes whose promoters are associated with early seed and embryo development include rice *glutelin* ("GluA-3," Yoshihara and Takaiwa, 1996, Plant Cell Physiol 37:107-11; "GluB-1," Takaiwa et al., 1996, Plant Mol Biol 30:1207-21; Washida et al., 1999, Plant Mol Biol 40:1-12; "Gt3," Leisy et al., 1990, Plant Mol Biol 14:41-50), rice *prolamin* (Zhou & Fan, 1993, Transgenic Res 2:141-6), wheat *prolamin* (Hammond-Kosack et al., 1993, EMBO J 12:545-54), maize *zein* (Z4, Matzke et al., 1990, Plant Mol Biol 14:323-32), and barley *B-hordeins* (Entwistle et al., 1991, Plant Mol Biol 17:1217-31). Other genes whose promoters are associated with early seed and embryo development include oil palm GLO7A (7S globulin, Morcillo et al., 2001, Physiol Plant 112:233-243), *Brassica napus napin*, 2S storage protein, and napA gene (Josefsson et al.,

15

1987, J Biol Chem 262:12196-201; Stalberg et al., 1993, Plant Mol Biol 1993 23:671-83; Ellerstrom et al., 1996, Plant Mol Biol 32:1019-27), *Brassica napus oleosin* (Keddie et al., 1994, Plant Mol Biol 24:327-40), *Arabidopsis oleosin* (Plant et al., 1994, Plant Mol Biol 25:193-205), *Arabidopsis* FAE1 (Rossak et al., 2001, Plant Mol Biol 46:717-25),

5   *Canavalia gladiata* conA (Yamamoto et al., 1995, Plant Mol Biol 27:729-41), and *Catharanthus roseus* strictosidine synthase (Str, Ouwerkerk and Memelink, 1999, Mol Gen Genet 261:635-43). In another preferred embodiment, regulatory sequences from genes expressed during oil biosynthesis are used (see, e.g., US Pat No: 5,952, 544). Alternative promoters are from plant storage protein genes (Bevan et al, 1993, Philos

10   Trans R Soc Lond B Biol Sci 342:209-15).

         In yet another aspect, in some cases it may be desirable to inhibit the expression of endogenous HIO30 in a host cell. Exemplary methods for practicing this aspect of the invention include, but are not limited to antisense suppression (Smith, *et al., Nature* 334:724-726, 1988; van der Krol et al., Biotechniques (1988) 6:958-976); co-suppression

15   (Napoli, *et al, Plant Cell* 2:279-289, 1990); ribozymes (PCT Publication WO 97/10328); and combinations of sense and antisense (Waterhouse, *et al., Proc. Natl. Acad. Sci. USA* 95:13959-13964, 1998). Methods for the suppression of endogenous sequences in a host cell typically employ the transcription or transcription and translation of at least a portion of the sequence to be suppressed. Such sequences may be homologous to coding as well

20   as non-coding regions of the endogenous sequence. Antisense inhibition may use the entire cDNA sequence (Sheehy et al., Proc. Natl. Acad. Sci. USA (1988) 85:8805-8809), a partial cDNA sequence including fragments of 5' coding sequence, (Cannon et al., Plant Molec. Biol. (1990) 15:39-47), or 3' non-coding sequences (Ch'ng et al., Proc. Natl. Acad. Sci. USA (1989) 86:10006-10010). Cosuppression techniques may use the entire cDNA

25   sequence (Napoli et al., *supra*; van der Krol et al., The Plant Cell (1990) 2:291-299) or a partial cDNA sequence (Smith et al., Mol. Gen. Genetics (1990) 224:477-481).

         Standard molecular and genetic tests may be performed to further analyze the association between a gene and an observed phenotype. Exemplary techniques are

30   described below.

         1. <u>DNA/RNA analysis</u>

         The stage- and tissue-specific gene expression patterns in mutant versus wild-type lines may be determined, for instance, by in situ hybridization. Analysis of the methylation status of the gene, especially flanking regulatory regions, may be performed. Other

suitable techniques include overexpression, ectopic expression, expression in other plant species and gene knock-out (reverse genetics, targeted knock-out, viral induced gene silencing [VIGS, see Baulcombe D, *Arch Virol Suppl* 15:189-201, 1999]).

5   In a preferred application expression profiling, generally by microarray analysis, is used to simultaneously measure differences or induced changes in the expression of many different genes. Techniques for microarray analysis are well known in the art (Schena M *et al.*, Science (1995) 270:467-470; Baldwin D *et al.*, *Cur Opin Plant Biol.* 2(2):96-103, 1999; Dangond F, Physiol Genomics (2000) 2:53-58; van Hal NL *et al.*, J Biotechnol (2000) 78:271-280; Richmond T and Somerville S, Curr Opin Plant Biol (2000) 3:108-

10   116). Expression profiling of individual tagged lines may be performed. Such analysis can identify other genes that are coordinately regulated as a consequence of the overexpression of the gene of interest, which may help to place an unknown gene in a particular pathway.

          2. Gene Product Analysis

15   Analysis of gene products may include recombinant protein expression, antisera production, immunolocalization, biochemical assays for catalytic or other activity, analysis of phosphorylation status, and analysis of interaction with other proteins via yeast two-hybrid assays.

          3. Pathway Analysis

20   Pathway analysis may include placing a gene or gene product within a particular biochemical, metabolic or signaling pathway based on its mis-expression phenotype or by sequence homology with related genes. Alternatively, analysis may comprise genetic crosses with wild-type lines and other mutant lines (creating double mutants) to order the gene in a pathway, or determining the effect of a mutation on expression of downstream

25   "reporter" genes in a pathway.

Generation of Mutated Plants with an Altered Oil Content Phenotype

          The invention further provides a method of identifying plants that have mutations in endogenous HIO30 that confer altered oil content, and generating altered oil content

30   progeny of these plants that are not genetically modified. In one method, called "TILLING" (for targeting induced local lesions in genomes), mutations are induced in the seed of a plant of interest, for example, using EMS treatment. The resulting plants are grown and self-fertilized, and the progeny are used to prepare DNA samples. HIO30-specific PCR is used to identify whether a mutated plant has a HIO30 mutation. Plants

having HIO30 mutations may then be tested for altered oil content, or alternatively, plants may be tested for altered oil content, and then HIO30-specific PCR is used to determine whether a plant having altered oil content has a mutated HIO30 gene. TILLING can identify mutations that may alter the expression of specific genes or the activity of proteins
5   encoded by these genes (see Colbert et al (2001) Plant Physiol 126:480-484; McCallum et al (2000) Nature Biotechnology 18:455-457).

In another method, a candidate gene/Quantitative Trait Locus (QTLs) approach can be used in a marker-assisted breeding program to identify alleles of or mutations in the HIO30 gene or orthologs of HIO30 that may confer altered oil content (see Bert et al.,
10   Theor Appl Genet. 2003 Jun;107(1):181-9; and Lionneton et al, Genome. 2002 Dec;45(6):1203-15). Thus, in a further aspect of the invention, a HIO30 nucleic acid is used to identify whether a plant having altered oil content has a mutation in endogenous HIO30 or has a particular allele that causes altered oil content.

All publications cited herein are expressly incorporated herein by reference for the
15   purpose of describing and disclosing compositions and methodologies that might be used in connection with the invention. All cited patents, patent applications, and sequence information in referenced websites and public databases are also incorporated by reference.

20   **EXAMPLES**

EXAMPLE 1

Generation of Plants with a HIO30 Phenotype by Transformation with an Activation Tagging Construct

Mutants were generated using the activation tagging "ACTTAG" vector, pSKI015
25   (GI#6537289; Weigel D *et al., supra*). Standard methods were used for the generation of *Arabidopsis* transgenic plants, and were essentially as described in published application PCT WO0183697. Briefly, T0 *Arabidopsis* (Col-0) plants were transformed with *Agrobacterium* carrying the pSKI015 vector, which comprises T-DNA derived from the *Agrobacterium* Ti plasmid, an herbicide resistance selectable marker gene, and the 4X
30   CaMV 35S enhancer element. Transgenic plants were selected at the T1 generation based on herbicide resistance. T2 seed was collected from T1 plants and stored in an indexed collection, and a portion of the T2 seed was accessed for the screen.

Quantitative determination of seed fatty acid content was performed using the follows methods. An aliquot of 15 to 20 T2 seeds from each line tested, which generally

contained homozygous insertion, homozygous wild-type, and heterozygous genotypes in a standard 1:1:2 ratio, was massed on UMT-2 ultra-microbalance (Mettler-Toledo Co., Ohio, USA) and then transferred to a glass extraction vial. Whole seeds were trans-esterified in 500 ul 2.5% H_2SO_4 in MeOH for 3 hours at 80 °C, following the method of

5   Browse et al. (Biochem J 235:25-31, 1986) with modifications. A known amount of heptadecanoic acid was included in the reaction as an internal standard. 750 ul of water and 400 ul of hexane were added to each vial, which was then shaken vigorously and allowed to phase separate. Reaction vials were loaded directly onto GC for analysis and the upper hexane phase was sampled by the autosampler. Gas chromatography with

10   Flame Ionization detection was used to separate and quantify the fatty acid methyl esters. Agilent 6890 Plus GC's were used for separation with Agilent Innowax columns (30m x 0.25mm ID, 250um film thickness). The carrier gas was Hydrogen at a constant flow of 2.5 ml/ minute. 1ul of sample was injected in splitless mode (inlet temperature 220°C, Purge flow 15ml/min at 1 minute). The oven was programmed for an initial temperature

15   of 105°C, initial time 0.5 minutes, followed by a ramp of 60°C per minute to 175°C, a 40°C /minute ramp to 260°C with a final hold time of 2 minutes. Detection was by Flame Ionization (Temperature 275°C, Fuel flow 30.0 ml/min, Oxidizer 400.0 ml/min). Instrument control and data collection and analysis was using the Millennium Chromatography Management System (Version 3.2, Waters Corporation, Milford, MA).

20   Integration and quantification were performed automatically, but all analyses were subsequently examined manually to verify correct peak identification and acceptable signal to noise ratio before inclusion of the derived results in the study.

The ACTTAG line designated W000086431 was identified as having a high oil phenotype. Specifically, oil constituted 34.8% of seed mass (w/w) compared to an

25   average oil content of 28.7% of other ACTTAG lines grown and analyzed in the same conditions (i.e. reference lines). Reanalysis of the same seed was performed in triplicate. Oil constituted 32.1% of seed mass, confirming an increase in oil content relative to reference.

The W000086431 line has three loci of T-DNA. One locus, was unlinked to the

30   phenotype, and two closely linked loci were identified which cosegregated with the high oil phenotype. T2 individuals homozygous for the high oil loci produced seed with an oil content of 115.4% of the reference, T2 individuals hemizygous for the loci produced seed with an oil content of 118.4% of the reference. T2 individuals lacking the HIO-30 locus had oil contents of 105% of the reference. Because the homozygotes and hemizygotes for

the high oil loci display a similar increase in oil content, it was determined that the high oil phenotype of W000068431 is dominant.

EXAMPLE 2

5   Characterization of the T-DNA Insertion in Plants Exhibiting the Altered Oil Content Phenotype.

We performed standard molecular analyses, essentially as described in patent application PCT WO0183697, to determine the site of the T-DNA insertion associated with the altered oil content phenotype. Briefly, genomic DNA was extracted from plants

10   exhibiting the altered oil content phenotype. PCR, using primers specific to the pSKI015 vector, confirmed the presence of the 35S enhancer in plants from line W000086431, and Southern blot analysis verified the genomic integration of the ACTTAG T-DNA.

Plasmid rescue and/or inverse PCR were used to recover genomic DNA flanking the T-DNA insertion, which was then subjected to sequence analysis using a basic

15   BLASTN search and/or a search of the Arabidopsis Information Resource (TAIR) database (available at the arabidopsis.org website). Seeds from 18 T3 families descended from the mutant were harvested. The seed oil content of these families was determined as described in Example 1. The genotypes of these families with respect to a T-DNA insert were determined by T–DNA specific PCR using primers that are specific to one of the

20   corresponding genomic region. The average oil content of T3 families containing the T-DNA insert at locus 2 and 3 was higher than those families lacking the insert at the corresponding loci. Therefore, we concluded that locus 2 and/or 3 is linked to the high oil phenotype. By contrast, the average oil content of T3 families containing the T-DNA insert at locus 1 was lower than /approximately the same as those families lacking the

25   insert at the corresponding locus, and we concluded that locus 1 is not linked to the high oil phenotype.

Sequence analysis revealed that the start codon of the nucleotide sequence presented as SEQ ID NO: 1, which we designated HIO30, was approximately 5.6 kb 5' of the upstream border of the T-DNA insert at locus 3.

30

EXAMPLE 3

Analysis of *Arabidopsis* HIO30 Sequence

Sequence analyses were performed with BLAST (Altschul *et al.*, 1997, J. Mol. Biol. 215:403-410), PFAM (Bateman *et al.*, 1999, Nucleic Acids Res 27:260-262),

PSORT (Nakai K, and Horton P, 1999,Trends Biochem Sci 24:34-6), and/or CLUSTAL (Thompson JD et al, 1994, Nucleic Acids Res 22:4673-4680).

BLASTN of SEQ ID NO:1 against GenBank sequences identified 6 Arabidopsis ESTs derived from cDNA clones derived from the same genomic region as At3g52260.

5   This gene appears to occur as a single copy in Arabidopsis.

BLASTN also identified additional ESTs from other plant species. The following three potato (Solanum tuberosum) ESTs were identified as candidate orthologs; BLAST scores are in parentheses: GI#s 13614224 (1054 4.2e-41), 18257466 (1031 4.8e-40), and 14266906 (1140 7.2e-50). Other plant ESTs were sorted by species, and then assembled

10 into the least number of contigs, represented by SEQ ID NOs 3-6, and described further below. In most cases, the EST contigs represent partial coding regions. But, the entire cDNA sequence of the ortholog can be determined by someone skilled in molecular biology techniques.

SEQ ID NO:3 is from tomato (Lycoprsicon esculentum), and is a contig of the

15 following sequences: GI#s 5894121, 9503437, 5894459, and 5889410. It has 56% identity with SEQ ID NO:1.

SEQ ID NO:4 is from soy (Glycine max), and is a contig of GI#s 6913831, and 5760781. It has 66% identity with SEQ ID NO:1.

SEQ ID NO:5 is from corn (Zea mays), and is a contig of GI#s 21215230,

20 5268759, and 22521540. It has 57% identity with SEQ ID NO:1.

SEQ ID NO:6 is from wheat (Triticum aestivum) and is a contig of GI#s 19955658 and 9364987. It has 62% identity with SEQ ID NO:1.

BLASTP analysis returned redundant entries for the At3g52260 gene product. The only other hits returned were numerous bacterial gene products corresponding to 23S

25 RNA pseudouridylate synthase. No other plant sequences were returned.

PFam analysis detected an RNA pseudouridylate synthase domain (PF00849). In accordance with this functional prediction, PSORT2 predicts that the At3g52260 gene product is cytoplasmic (44%). The gene may have a regulatory function in the expression of genes involved in fatty acid metabolism or related pathways.

30

EXAMPLE 4

Confirmation of Phenotype/Genotype Association

RT-PCR analysis showed that the HIO30 gene was overexpressed in plants from the line displaying the HIO30 phenotype. Specifically, RNA was extracted from rosette leaves

and/or siliques of plants exhibiting the HIO30 phenotype collected at a variety of developmental stages and pooled. RT-PCR was performed using primers specific to the sequence presented as SEQ ID NO:1, to other predicted genes in the vicinity of the T-DNA insertion, and to a constitutively expressed actin gene (positive control). The results

5   showed that plants displaying the HIO30 phenotype over-expressed the mRNA for the HIO30 gene, indicating the enhanced expression of the HIO30 gene is correlated with the HIO30 phenotype.

The dominant inheritance pattern of the HIO30 phenotype is confirmed through genetic analysis. In general, genetic analysis involves the production and analysis of F1

10   hybrids. Typically, F1 crosses are carried out by collecting pollen from T2 plants, which is used to pollinate wild type plants. Such crosses are carried out by taking approximately 4 flowers from each selected individual plants, and using the T2 flower as the male pollen donor and flowers of the wild type plants as the female. 4-5 crosses are done for an individual of interest. Seed formed from crosses of the same individual are pooled,

15   planted and grown to maturity as F1 hybrids.

IT IS CLAIMED:

1. A transgenic plant comprising a plant transformation vector comprising a nucleotide sequence that encodes or is complementary to a sequence that encodes a HIO30

5   polypeptide comprising the amino acid sequence of SEQ ID NO:2, or an ortholog thereof, whereby the transgenic plant has a high oil phenotype relative to control plants.

2. The transgenic plant of Claim 1, which is selected from the group consisting of rapeseed, soy, corn, sunflower, cotton, cocoa, safflower, oil palm, coconut palm, flax,

10   castor and peanut.

3. A plant part obtained from the plant according to Claim 1.

4. The plant part of Claim 3, which is a seed.

15

5. A method of producing oil comprising growing the transgenic plant of Claim1 and recovering oil from said plant.

6. A method of producing a high oil phenotype in a plant, said method comprising:

20   a)   introducing into progenitor cells of the plant a plant transformation vector comprising a nucleotide sequence that encodes or is complementary to a sequence that encodes a HIO30 polypeptide comprising the amino acid sequence of SEQ ID NO:2, or an ortholog thereof, and

        b)   growing the transformed progenitor cells to produce a transgenic plant, wherein

25   said polynucleotide sequence is expressed, and said transgenic plant exhibits an altered oil content phenotype relative to control plants.

7. A plant obtained by a method of Claim 6.

30   8. The plant of Claim 7, which is selected from the group consisting of rapeseed, soy, corn, sunflower, cotton, cocoa, safflower, oil palm, coconut palm, flax, castor and peanut.

9. A method of generating a plant having a high oil phenotype comprising identifying a plant that has an allele in its HIO30 gene that results in increased oil content compared to

plants lacking the allele and generating progeny of said identified plant, wherein the generated progeny inherit the allele and have the high oil phenotype.

10. The method of Claim 9 that employs candidate gene/QTL methodology.

5

11. The method of Claim 9 that employs TILLING methodology.

SEQUENCE LISTING

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<120>   GENERATION OF PLANTS WITH ALTERED OIL CONTENT

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Trp Arg Asp Ser Pro Ser Ser His His Val Pro Arg Glu Phe Ser Ser
            35   40   45

Arg Leu Gly Ser Gly Asp Phe Arg Arg Pro Ser Ser Leu Thr Gln Leu
    50   55   60

Leu Arg Leu Ile Gly Ser Glu Leu Met Arg Leu Leu Phe Lys Gly Ala
65   70   75   80

Ile Phe Asn Thr Gln Gly Gly Arg His Gln Phe Val Glu Glu Thr Ser
                85   90   95

His Gly Tyr Thr Ser Ser Arg Ser Ser Ala Arg Met Phe Asp Asn Tyr
            100   105   110

Arg Pro Ser Ala Ser Arg Gly Asp Trp Arg Tyr Thr Arg Asn Cys Arg
            115   120   125

Asp Asp Arg Val Ser Val Ser Gln Lys Glu Trp Lys Cys Asn Thr Trp
    130   135   140

Glu Met Ser Asn Gly Ser Ser Arg Ser Phe Glu Arg Pro Phe Gly Ile
145   150   155   160

Arg Asn Gly Arg Arg Ser Val Asp Glu Arg Pro Leu His Ala Ser Asp
                165   170   175

Thr His Ser Thr Val Val Asn Ser Leu Asp Pro Ala Asn Ser Ala His
            180   185   190

Tyr Leu Asp Asn Glu Ile Ser Thr Pro Val Arg Ser Leu Lys Ile Lys
            195   200   205

4

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Asn Glu His Lys Phe Ser Asp Gln Arg Leu Ser Leu Pro Ser Asp Pro
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His Ser Glu Cys Ile Ser Leu Phe Glu Arg Pro Ser Ser Glu Asn Asn
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Tyr Gly Asn Lys Val Cys Ser Pro Ala Lys Gln Cys Asn Asp Leu Met
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Tyr Gly Arg Arg Leu Val Ser Asp Asn Ser Leu Asp Ala Pro Ile Pro
            260             265             270

Asn Ala Glu Leu Glu Gly Thr Trp Glu Gln Leu Arg Leu Lys Asp Pro
            275             280             285

Gln Asp Asn Asn Ser Leu His Gly Ile Asn Asp Ile Asp Gly Asp Arg
    290             295             300

Lys Cys Ala Lys Glu Ser Ser Leu Gly Ala Thr Gly Lys Leu Pro Leu
305             310             315             320

Trp Asn Ser Ser Gly Ser Phe Ala Ser Gln Ser Ser Gly Phe Ser His
                325             330             335

Ser Ser Ser Leu Lys Ser Leu Gly Ala Val Asp Ser Ser Asp Arg Lys
            340             345             350

Ile Glu Val Leu Pro Lys Ile Val Thr Val Thr Gln Ser Ser Ser Gly
            355             360             365

Asp Ala Thr Ala Cys Ala Thr Thr Thr His Leu Ser Glu Glu Met Ser
    370             375             380

Ser Arg Lys Lys Gln Arg Leu Gly Trp Gly Glu Gly Leu Ala Lys Tyr
385             390             395             400

Glu Lys Lys Lys Val Asp Val Asn Pro Asn Glu Asp Gly Thr Thr Leu
            405             410             415

Met Glu Asn Gly Leu Glu Glu Leu His Ser Leu Asn Lys Asn Ile Ala
            420             425             430

Asp Lys Ser Pro Thr Ala Ala Ile Val Pro Asp Tyr Gly Ser Pro Thr
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Thr Pro Ser Ser Val Ala Cys Ser Ser Ser Pro Gly Phe Ala Asp Lys
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5

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                450                 455                      460


    Ser Ser Pro Lys Ala Ala Ile Ala Ala Ser Asp Val Ser Asn Met Cys
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    Arg Ser Pro Ser Pro Val Ser Ser Ile His Leu Glu Arg Phe Pro Ile
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    Asn Ile Glu Glu Leu Asp Asn Ile Ser Met Glu Arg Phe Gly Cys Leu
                500             505             510


    Leu Asn Glu Leu Leu Gly Thr Asp Asp Ser Gly Thr Gly Asp Ser Ser
                515             520             525


    Ser Val Gln Leu Thr Ser Met Asn Thr Leu Leu Ala Trp Lys Gly Glu
    530             535             540


    Ile Leu Lys Ala Val Glu Met Thr Glu Ser Glu Ile Asp Leu Leu Glu
    545             550             555                      560


    Asn Lys His Arg Thr Leu Lys Leu Glu Gly Arg Arg His Ser Arg Val
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    Val Gly Pro Ser Ser Tyr Cys Cys Asp Gly Asp Ala Asn Val Pro Lys
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    Glu Gln Ala Ser Cys Ser Leu Asp Pro Lys Ala Thr Ala Ser Ser Val
                595             600             605


    Ala Lys Thr Leu Val Arg Ala Pro Val His Gln Ala Gly Leu Ala Lys
        610             615             620


    Val Pro Ala Asp Val Phe Glu Asp Ser Pro Gly Glu Val Lys Pro Leu
    625             630             635                      640


    Ser Gln Ser Phe Ala Thr Val Glu Arg Glu Glu Asp Ile Leu Pro Ile
                    645             650                      655


    Pro Ser Met Lys Ala Ala Val Ser Ser Lys Glu Ile Asn Thr Pro Ala
                660             665             670


    Phe Ala Asn Gln Glu Thr Ile Glu Val Ser Ser Ala Asp Asp Ser Met
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    Ala Ser Lys Glu Asp Leu Phe Trp Ala Lys Leu Leu Ser Ala Asn Lys
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Lys Tyr Ala Cys Glu Ser Ser Gly Val Phe Asn Gln Leu Leu Pro Arg
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Asp Phe Asn Ser Ser Asp Asn Ser Arg Phe Pro Gly Ile Cys Gln Thr
            725             730             735

Gln Phe Asp Ser His Val Gln Glu Lys Ile Ala Asp Arg Val Gly Leu
            740             745             750

Leu Arg Ala Arg Glu Lys Ile Leu Leu Leu Gln Phe Lys Ala Phe Gln
        755             760             765

Leu Ser Trp Lys Lys Asp Leu Asp Gln Leu Ala Leu Ala Lys Tyr Gln
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Ser Lys Ser Ser Lys Lys Thr Glu Leu Tyr Pro Asn Ala Lys Asn Gly
785             790             795             800

Gly Tyr Leu Lys Leu Pro Gln Ser Val Arg Leu Arg Phe Ser Ser Ser
                805             810             815

Ala Pro Arg Arg Asp Ser Val Val Pro Thr Thr Glu Leu Val Ser Tyr
            820             825             830

Met Glu Lys Leu Leu Pro Gly Thr His Leu Lys Pro Phe Arg Asp Ile
        835             840             845

Leu Lys Met Pro Ala Met Ile Leu Asp Glu Lys Glu Arg Val Met Ser
    850             855             860

Arg Phe Ile Ser Ser Asn Gly Leu Ile Glu Asp Pro Cys Asp Val Glu
865             870             875             880

Lys Glu Arg Thr Met Ile Asn Pro Trp Thr Ser Glu Glu Lys Glu Ile
            885             890             895

Phe Leu Asn Leu Leu Ala Met His Gly Lys Asp Phe Lys Lys Ile Ala
        900             905             910

Ser Ser Leu Thr Gln Lys Thr Thr Ala Asp Cys Ile Asp Tyr Tyr Tyr
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Lys Asn His Lys Ser Asp Cys Phe Gly Lys Ile Lys Lys Gln Arg Ala
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Tyr Gly Lys Glu Gly Lys His Thr Tyr Met Leu Ala Pro Arg Lys Lys
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Trp Lys Arg Glu Met Gly Ala Ala Ser Leu Asp Ile Leu Gly Asp Val
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Ser Ile Ile Ala Ala Asn Ala Gly Lys Val Ala Ser Thr Arg Pro Ile
                980                     985                 990


Ser Ser Lys Lys Ile Thr Leu Arg  Gly Cys Ser Ser Ala  Asn Ser Leu
                995                 1000                1005


Gln His  Asp Gly Asn Asn Ser  Glu Gly Cys Ser Tyr  Ser Phe Asp
     1010                 1015                1020


Phe Pro  Arg Lys Arg Thr Ala  Gly Ala Asp Val Leu  Ala Val Gly
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Pro Leu  Ser Pro Glu Gln Ile  Asn Ser Cys Leu Arg  Thr Ser Val
     1040                 1045                1050


Ser Ser  Arg Glu Arg Cys Met  Asp His Leu Lys Phe  Asn His Val
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Val Lys  Lys Pro Arg Ile Ser  His Thr Leu His Asn  Glu Asn Ser
     1070                 1075                1080


Asn Thr  Leu His Asn Glu Asn  Ser Asn Glu Glu Asp  Asp Ser Cys
     1085                 1090                1095


Ser Glu  Glu Ser Cys Gly Glu  Thr Gly Pro Ile His  Trp Thr Asp
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Asp Glu  Arg Ser Ala Phe Ile  Gln Gly Phe Ser Leu  Phe Gly Lys
     1115                 1120                1125


Asn Phe  Ala Ser Ile Ser Arg  Tyr Val Gly Thr Arg  Ser Pro Asp
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Gln Cys  Lys Val Phe Phe Ser  Lys Val Arg Lys Cys  Leu Gly Leu
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Glu Ser  Ile Lys Phe Gly Ser  Gly Asn Val Ser Thr  Ser Val Ser
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Val Asp  Asn Gly Asn Glu Gly  Gly Gly Ser Asp Leu  Glu Asp Pro
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Cys Pro Met Glu Ser Asn Ser Gly Ile Val Asn Asn Gly Val Cys
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Ala Lys Met Gly Met Asn Ser Pro Thr Ser Pro Phe Asn Met Asn
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Gln Asp Gly Val Asn Gln Ser Gly Ser Ala Asn Val Lys Ala Asp
    1220   1225   1230

Leu Ser Arg Ser Glu Glu Glu Asn Gly Gln Lys Tyr Leu Cys Leu
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Lys Asp Asp Asn Asn Leu Val Asn Asn Ala Tyr Val Asn Gly Gly
    1250   1255   1260

Phe Pro Ser Leu Val Ser Glu Ser Cys Arg Asp Leu Val Asp Ile
    1265   1270   1275

Asn Thr Val Glu Ser Gln Ser Gln Ala Ala Gly Lys Ser Lys Ser
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Asn Asp Leu Met Ser Met Glu Ile Asp Glu Gly Val Leu Thr Ser
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Val Thr Ile Ser Ser Glu Pro Leu Tyr Cys Gly Leu Ser Val Leu
    1310   1315   1320

Ser Asn Val Ile Val Glu Thr Pro Thr Glu Ile Ser Arg Lys Gly
    1325   1330   1335

Ser Gly Asp Gln Gly Ala Thr Met Pro Lys Phe Ser Ser Lys Asn
    1340   1345   1350

Gln Asp Gly Val Met Gln Ala Ala Asn Arg Thr Arg Asn Ser Gly
    1355   1360   1365

Leu Glu Pro Glu Ser Ala Pro Ser Gly Phe Arg Tyr Pro Glu Cys
    1370   1375   1380

Leu His His Val Pro Ile Glu Val Cys Thr Glu Asn Pro Ile Gly
    1385   1390   1395

Val Ser Ala Pro Arg Gly Asn Pro Asn Cys His Ala Glu Ser Glu
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Ser Gly Asn Ser Leu Val Gly Gln Val Asp Glu Thr His Asp Leu

9

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Gly Trp  Pro Lys Asn Asn Leu  Glu Leu Asp Gly Arg  Leu Gln Val
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Leu Gly  His Val Asn Pro Glu  Gln Ile Gly Leu Leu  Lys Ala Thr
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Asn Thr  Glu Ser Cys Gln Asn  Pro Gln Arg Ser Val  Thr Gln Asp
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Leu Ser  Arg Ile Ser Arg Ser  Lys Ser Asp Leu Ile  Val Lys Thr
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Gln Arg  Thr Gly Glu Gly Phe  Ser Leu Thr Lys Cys  Thr Ser Ser
    1490                   1495                   1500


Ala Pro  Lys Pro Leu Ala Val  Ser His Lys Glu Gly  Arg Ser Gly
    1505                   1510                   1515


His Ser  Arg Ser His Ser Phe  Ser Leu Ser Asp Thr  Glu Arg Leu
    1520                   1525                   1530


His Lys  Asn Gly Asp Val Lys  Leu Phe Gly Thr Val  Leu Thr Thr
    1535                   1540                   1545


Asp Glu  Asn Gly Ile Lys Gln  Lys His Asn Pro Cys  Gly Ile Val
    1550                   1555                   1560


Arg Ser  Ser Ser Thr Leu Ser  Arg Asp His Asp Thr  Arg His His
    1565                   1570                   1575


Tyr Ile  Asn Gln Gln His Leu  Gln Asn Val Pro Ile  Thr Ser Tyr
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Gly Phe  Trp Asp Gly Asn Arg  Ile Gln Thr Gly Leu  Thr Ser Leu
    1595                   1600                   1605


Pro Glu  Ser Ala Lys Leu Leu  Ala Ser Cys Pro Glu  Ala Phe Ser
    1610                   1615                   1620


Thr His  Leu Lys Gln Gln Val  Gly Asn Ser Lys Glu  Ile Leu Val
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Asp Val  Asn Gly Gly Ile Leu  Ser Phe Gly Lys His  Asn Glu Asp
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10

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12

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